



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Retargeting FX binding-ablated HAdV-5 to vascular cells by inclusion of the RGD-4C peptide in hexon hypervariable region 7 and the HI loop

Citation for published version:

Robertson, S, Parker, AL, Clarke, C, Duffy, MR, Alba, R, Nicklin, SA & Baker, AH 2016, 'Retargeting FX binding-ablated HAdV-5 to vascular cells by inclusion of the RGD-4C peptide in hexon hypervariable region 7 and the HI loop', *Journal of General Virology*. <https://doi.org/10.1099/jgv.0.000505>

Digital Object Identifier (DOI):

[10.1099/jgv.0.000505](https://doi.org/10.1099/jgv.0.000505)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Journal of General Virology

Publisher Rights Statement:

This is an open access article published by the Microbiology Society under the Creative Commons Attribution License

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Journal of General Virology

Retargeting FX binding-ablated HAdV-5 to vascular cells by inclusion of the RGD-4C peptide in hexon hypervariable region 7 and the HI loop --Manuscript Draft--

Manuscript Number:	JGV-D-16-00091R1
Full Title:	Retargeting FX binding-ablated HAdV-5 to vascular cells by inclusion of the RGD-4C peptide in hexon hypervariable region 7 and the HI loop
Short Title:	Retargeting FX-ablated HAdV-5 by RGD-4C peptide insertions
Article Type:	Short Communication
Section/Category:	Animal - Large DNA Viruses
Corresponding Author:	Andrew H Baker, PhD University of Edinburgh UNITED KINGDOM
First Author:	Stacy Robertson, PhD
Order of Authors:	Stacy Robertson, PhD
	Alan L Parker, PhD
	Carolyn Clarke, PhD
	Margaret R Duffy, PhD
	Raul Alba, PhD
	Stuart A Nicklin, PhD
	Andrew H Baker, PhD
Abstract:	Recent studies have generated interest into the function of human adenovirus serotype 5 (HAdV-5) hexon: factor X (FX)-binding and subsequent hepatocyte transduction and interaction with the immune system. Here, we retargeted adenovirus serotype 5 vectors, ablated for FX interaction, by replacing amino acids in hexon HVR7 with RGD-4C or inserting the peptide into the fiber HI loop. These genetic modifications in the capsid were compatible with virus assembly, and could efficiently retarget transduction of the vector via the $\alpha\beta 3/5$ integrin-mediated pathway, but did not alter immune recognition by pre-existing human neutralising anti-HAdV-5 antibodies or by natural antibodies in mouse serum. Thus, FX-binding ablated HAdV-5 can be retargeted but remain sensitive to immune-mediated attack. These findings further refine HAdV-5 based vectors for human gene therapy and inform future vector development.

Retargeting FX binding-ablated HAdV-5 to vascular cells by inclusion of the RGD-4C peptide in hexon hypervariable region 7 and the HI loop

Running title: *Retargeting FX-ablated HAdV-5 by RGD-4C peptide insertions*

Stacy Robertson^{1*}

Alan L. Parker^{1,2 *}

Carolyn Clarke¹

Margaret R. Duffy^{1,3}

Raul Alba^{1,4}

Stuart A. Nicklin¹

Andrew H. Baker^{1,5}

¹ British Heart Foundation Glasgow Cardiovascular Research Centre, University of Glasgow, Glasgow, United Kingdom.

² Present address: Division of Cancer & Genetics, Henry Wellcome Building, Cardiff University School of Medicine, Heath Park, Cardiff, CF14 4XN. United Kingdom.

³ Present address: Department of Oncology, University of Oxford, Headington, Oxford, United Kingdom.

⁴ Present address: VCN Biosciences, Avinguda de la Generalitat, Sant Cugat del Vallès, Barcelona, Spain.

⁵ Present Address: Centre for Cardiovascular Science, University of Edinburgh, Queen's Medical Research Institute, Edinburgh, United Kingdom.

* Equal contribution

Corresponding author: Professor Andrew Baker

Address: Centre for Cardiovascular Science
University of Edinburgh
Queen's Medical Research Institute
47 Little France Crescent,
Edinburgh,
United Kingdom.
EH16 4TJ

Email: andy.baker@ed.ac.uk

Telephone: +44 (0) 1312426728

Keywords: gene therapy, adenovirus, coagulation factor X, targeting, hexon, fiber

Word count: 1,903

Abstract

Recent studies have generated interest into the function of human adenovirus serotype 5 (HAd-V5) hexon: factor X (FX)-binding and subsequent hepatocyte transduction and interaction with the immune system. Here, we retargeted adenovirus serotype 5 vectors, ablated for FX interaction, by replacing amino acids in hexon HVR7 with RGD-4C or inserting the peptide into the fiber HI loop. These genetic modifications in the capsid were compatible with virus assembly, and could efficiently retarget transduction of the vector via the $\alpha v\beta 3/5$ integrin-mediated pathway, but did not alter immune recognition by pre-existing human neutralising anti-HAdV-5 antibodies or by natural antibodies in mouse serum. Thus, FX-binding ablated HAdV-5 can be retargeted but remain sensitive to immune-mediated attack. These findings further refine HAd-V5 based vectors for human gene therapy and inform future vector development.

Adenoviral (Ad)-based vectors, particularly those based on human adenovirus serotype 5 (HAdV-5) are widely used clinically and experimentally. *In vitro*, and in local *in vivo* applications, HAdV-5 transduces cells via the human coxsackie and adenovirus receptor (hCAR) (Bergelson et al., 1997, Tomko et al., 1997). Our research has focussed on the use of adenoviral vectors as a tool for *ex vivo* manipulation of coronary artery bypass material to overexpress anti-proliferative genes (e.g. TIMP-3, p53) in coronary artery vascular smooth muscle cells (VSMCs), to prevent their migration, proliferation and formation of a neointimal lesion and ultimately graft reocclusion and failure following grafting (George et al., 2011). A significant limitation in this strategy is that VSMCs express a very low level of CAR and are thus refractory to transduction (Parker et al., 2013), necessitating high input titers of HAdV-5 to achieve therapeutic levels of transgene expression. For systemic *in vivo* applications, HAdV-5 efficiently and selectively transduces hepatocytes (Huard et al., 1995) in a process mediated through the engagement of the blood coagulation factor X (FX) with the hypervariable regions (HVR) of the HAdV-5 hexon protein (Hofherr et al., 2008, Kalyuzhniy et al., 2008, Waddington et al., 2008). Through selective modification of the HVRs, we previously generated a vector (HAdV-5T*) devoid of FX interactions and consequently hepatocyte transduction by introducing point mutations in key FX-interacting amino acids of the hexon protein HVR, namely T270P and E271G (HVR5) and I421G, T423N, E454S, L426Y and E451Q (all in HVR7) (Alba et al., 2009). Conversely, it has been reported that FX may actually offer a protective role in gene delivery, by shielding HAdV-5 from immune mediated attack by natural IgM and the classical complement system (Xu et al., 2013) that interact with HVRs, neutralising the virus (Ma et al., 2015). The specific amino acids responsible for immune recognition remain unknown, and the impact in humans remains unconfirmed. Incorporation of FX binding HVRs from HAdV-5 into HAdV-26 (non-FX binding Ad serotype) instilled liver transduction to this vector (Ma et al., 2015), reiterating the importance of FX in determining viral hepatic transduction. Therefore, retargeting of FX-ablated virus remains an area of interest for improving safety and efficacy of gene therapy vectors.

In this study, we evaluate optimal locations compatible for inserting targeting peptides within the HAdV-5T* vector. Previous studies identified the fiber HI loop as viable for peptide incorporation for retargeting *in vitro* and *in vivo* (Krasnykh et al., 1998, Dmitriev et al., 1998, Reynolds et al., 1999). Furthermore, the incorporation of RGD into HAdV-5 HVR5 was shown to result in increased transduction in non-permissive VSMC using a non-modified

HAdV-5 vector with high background hepatocyte transduction (Vigne et al., 1999). Here, three locations were selected for peptide incorporation: fiber HI loop (after amino acid 543G), and hexon HVRs 5 and 7 (Figs 1a - 1d). We selected the RGD-4C (CDCRGDCFC) peptide to test retargeting because it efficiently binds to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins expressed on many cell types, including endothelial cells (Zitzmann et al., 2002). This peptide has previously been widely used for adenoviral retargeting studies (Pasqualini et al., 1997, Dmitriev et al., 1998). The RGD-4C peptide was inserted into the HVRs with (designated “R” for replacement) or without (designated “I” for insertion) replacement of amino acids upstream of amino acid 272C (in HVR5) or 432K (in HVR7) whilst replacements involved removal of amino acids 272A–280L or 427T–435Q with simultaneous insertion of the peptide (Figs. 1c and 1d).

RGD-4C was cloned in modified shuttle plasmids containing the T* modified sequence (Fig. 1b) (Alba et al., 2009) or fiber HI loop (Fig. 1d) (Alba et al., 2010). Vectors were linearised and electroporated into BJ5183 bacteria cells with digested pAd5CMVlacZ for homologous recombination. Adenoviral production was performed in HEK293 cells as described previously (Alba et al., 2009). Peptide insertion in HAdV-5T*HVR7I proved incompatible with virus assembly, suggesting limitations for peptide insertion within this locale. Virus generation could be achieved for HAdV-5T*HVR5I, however titer assessment indicated very poor virus particle: plaque forming unit (vp: PFU) ratios (Fig. 1e), again suggesting simple insertion strategies within the HVRs appear to limit viral fitness, and this virus was therefore excluded from subsequent analysis. All other viruses were successfully propagated, verified by sequencing and quality control demonstrated consistent, high quality virus batches, as assessed by silver staining, BCA assay, nanoparticle tracking analysis (NanoSight LM10, Malvern) and plaque forming unit (pfu) assays (Figs. 1e and 1f), thus demonstrating amino acid removal from HVR5 and HVR7 are non-essential for virus assembly.

Recombinant viruses were evaluated for cell binding and transduction in α_v integrin-positive SKOV3 cells and A549 cells (Guo et al., 2009, Cannistra et al., 1995). Cells were transduced with HAdV-5, HAdV-5T* and peptide-modified adenoviral vectors at 50 pfu/cell, and transduction was assessed 48 hours post-transduction after a 3-hour exposure to each Ad by measuring β -galactosidase activity using the Tropix Galacton Plus and Tropix accelerator II kit (Applied Biosystems). Replacement of amino acids in HVR5 with RGD-4C or its insertion in the HI loop failed to increase cell transduction compared to HAdV-5 or HAdV-

5T*. However, replacing amino acids in HVR7 (HAdV-5T*HVR7R) with RGD4C in the HAdV-5T* background exhibited >10-fold increase in transduction compared to the parental HAdV-5T* vector (Figure 2a and 2b), validating HVR7 as a candidate site for targeting peptide insertion. Strategies to improve vascular tropism for effective gene delivery in coronary artery bypass graft purposes need to efficiently target VSMC, therefore we tested viral transduction in low passage (passage 2-5) human saphenous vein (HSV) primary VSMC, isolated as previously described (Southgate and Newby, 1990). Using the RGD targeted vectors (500 pfu/cell), we observed a robust 6-fold increase in transduction in HSV VSMC transduced with HAdV-5T*HVR7R or HAdV-5T*HI loop compared to HAdV-5 or HAdV-5T*, but not HAdV-5T*HVR5R (Fig. 2c). This contrasts with previous studies demonstrating insertion of RGD in HVR5 in an otherwise wild type HAdV-5 capsid, which did increase VSMC transduction (Vigne et al., 1999). This discrepancy could relate to the different RGD peptide sequence used in each study, our peptide contained 4 cysteine (CDCRGDCFC) in contrast to only two in the previous study (DCRGDCF) (Vigne et al., 1999). Additionally, these differences may relate to conformation alterations introduced by the specific combination of the FX-binding ablating mutations engineered in HVR5 combined with the RGD peptide incorporation.

Surface binding analysis was performed by incubating the recombinant vectors with the cells at 4°C for 1 hour. DNA was then isolated using QIAamp DNA Mini Kit (QIAGEN) and cell-membrane associated viral genomes were quantified by quantitative PCR as described previously (Alba et al., 2009). No change in binding was observed for HAdV-5T*HVR5R in any cell type, compared to HAdV-5T* (Figs. 2d, 2e, 2f), confirming that insertion of peptides within HVR5 confers little re-targeting benefit, whilst significant increases in cell association were observed for HAdV-5T*HVR7R (Figs. 2d and 2f). Increased binding to A549 cells was observed following peptide insertion in the fiber HI loop, although this did not correlate with increased transgene expression (Figs. 2b and 2e). In HSV VSMC, no increase in binding was observed for HAdV-5T*HI loop, however transduction was increased (Fig. 2f). This inconsistency could be due to differences in the ability of the virus to internalise and traffic through different cellular compartments following uptake. The discrepancy is observed across all of the cells type tested (SKOV3, A549 and HSV VSMC) indicating that the insertion of RGD-4C in the fiber HI loop may influence the surrounding capsid structure and hence effect trafficking to the nucleus. Further studies are required to fully delineate this finding.

FX binding HAdV-5 has been shown to prevent IgM and complement-mediated neutralization of the virus *in vivo* (Xu et al., 2013), with inhibitory serum components binding sites within the HAdV-5 HVRs (1-3 and 5-7) (Ma et al., 2015). We tested if this response was altered by the insertion of the RGD-4C peptide within these regions by investigating the sensitivity of the vectors to murine serum *in vitro* using a snake venom-derived factor X binding protein (X-bp) which binds to the Gla domain of FX preventing its interaction with Ad hexon (Waddington et al., 2008, Atoda et al., 1998). Recombinant Ad vectors (2×10^{10} vp/mL) were incubated with RPMI-1640 media, 90% C57BL/6 mouse serum or 90% C57BL/6 mouse serum pre-incubated with 40 μ g/mL X-bp, for 30 min at 37°C. Ad vector suspensions were diluted 200-fold in serum-free media and 100 μ L added to SKOV3 cells for 2 hours at 37°C before being replaced with RPMI-1640 media with 2% FCS. Transgene expression was quantified 16 hours post-transduction as relative light units (RLU) normalized to total protein. HAdV-5 mediated transduction significantly increased in the presence of serum, and reduced following pre-incubation of serum with X-bp (to bind and neutralize FX) (Waddington et al., 2008, Mizuno et al., 2001). HAdV-5T* demonstrated reduced transduction in the presence of murine serum compared to media alone (Fig. 3a). Peptide insertion failed to prevent virus neutralization and reduction in transduction, indicating that these sites are not critical to natural antibody mediated binding and neutralisation (Fig. 3a). To evaluate what effect, if any, peptide insertion might have on evasion of pre-existing human anti-HAdV-5 immunity, we performed neutralisation assays on HepG2 cells transduction following incubation with 1,000 vp/cell of Ad vectors in the presence of 1 IU/mL of FX and in 2.5% serum isolated from 103 cardiovascular patients (Parker et al., 2009). Reporter gene expression was quantified 48 hours post-transduction, and the changes in transduction relative to vector in the absence of serum was assessed (Fig. 3b). Incorporation of RGD-4C peptide into hexon or fiber had no discernible effect on evasion of pre-existing immunity, with 39.6% evasion observed at the 90% neutralisation level for both HAdV-5T*HVR7R and HAdV-5T*HI loop compared with 35.9% for the parental HAdV-5T* (Fig. 3b).

This study successfully retargeted a FX-ablated HAdV-5 vector to human smooth muscle cells and demonstrates that incorporation of the RGD-4C targeting peptide does not affect neutralisation by natural antibodies in murine sera or recognition by pre-existing anti-HAdV-5 immunity in the general population. Whilst ablation of FX interactions increases

neutralisation of Ad via IgM and complement, this has only been demonstrated in murine models to date (Xu et al., 2013). It remains unclear whether this is replicated in humans and this will be important to determine in the future. For intravascular delivery applications in humans, optimised retargeting strategies, including those based upon FX-binding ablated Ad vectors described herein, will be of key importance. Further studies will be required to evaluate these vectors in *ex vivo* human vein culture model (Soyombo et al., 1990) and suitable *in vivo* animal models to determine confirm the ability of these modified viruses to target to vasculature using clinically relevant model systems.

Acknowledgements

We thank Nicola Britton and Gregor Aitchison for technical assistance. This work was supported by the BBSRC (BB/I53297X/1). AHB is supported by the BHF chair of Translational Cardiovascular Sciences.

References

- ALBA, R., BRADSHAW, A. C., COUGHLAN, L., DENBY, L., MCDONALD, R. A., WADDINGTON, S. N., BUCKLEY, S. M., GREIG, J. A., PARKER, A. L., MILLER, A. M., WANG, H., LIEBER, A., VAN, R. N., MCVEY, J. H., NICKLIN, S. A. & BAKER, A. H. 2010. Biodistribution and retargeting of FX-binding ablated adenovirus serotype 5 vectors. *Blood*, 116, 2656-2664.
- ALBA, R., BRADSHAW, A. C., PARKER, A. L., BHELLA, D., WADDINGTON, S. N., NICKLIN, S. A., VAN, R. N., CUSTERS, J., GOUDSMIT, J., BAROUCH, D. H., MCVEY, J. H. & BAKER, A. H. 2009. Identification of coagulation factor (F)X binding sites on the adenovirus serotype 5 hexon: effect of mutagenesis on FX interactions and gene transfer. *Blood*, 114, 965-971.
- ATODA, H., ISHIKAWA, M., MIZUNO, H. & MORITA, T. 1998. Coagulation factor X-binding protein from Deinagkistrodon acutus venom is a Gla domain-binding protein. *Biochemistry*, 37, 17361-70.
- BERGELSON, J. M., CUNNINGHAM, J. A., DROGUETT, G., KURT-JONES, E. A., KRITHIVAS, A., HONG, J. S., HORWITZ, M. S., CROWELL, R. L. & FINBERG, R. W. 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science*, 275, 1320-1323.
- CANNISTRA, S. A., OTTENSMEIER, C., NILOFF, J., ORTA, B. & DICARLO, J. 1995. Expression and function of beta 1 and alpha v beta 3 integrins in ovarian cancer. *Gynecol Oncol*, 58, 216-25.
- DMITRIEV, I., KRASNYKH, V., MILLER, C. R., WANG, M., KASHENTSEVA, E., MIKHEEVA, G., BELOUSOVA, N. & CURIEL, D. T. 1998. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J. Virol.*, 72, 9706-9713.
- GEORGE, S. J., WAN, S., HU, J., MACDONALD, R., JOHNSON, J. L. & BAKER, A. H. 2011. Sustained reduction of vein graft neointima formation by ex vivo TIMP-3 gene therapy. *Circulation*, 124, S135-S142.
- GUO, L., ZHANG, F., CAI, Y. & LIU, T. 2009. Expression profiling of integrins in lung cancer cells. *Pathol Res Pract*, 205, 847-53.
- HOFHERR, S. E., SHASHKOVA, E. V., WEAVER, E. A., KHARE, R. & BARRY, M. A. 2008. Modification of adenoviral vectors with polyethylene glycol modulates in vivo tissue tropism and gene expression. *Mol. Ther.*, 16, 1276-1282.
- HUARD, J., LOCHMULLER, H., ACSADI, G., JANI, A., MASSIE, B. & KARPATI, G. 1995. The route of administration is a major determinant of the transduction efficiency of rat tissues by adenoviral recombinants. *Gene Ther.*, 2, 107-115.
- KALYUZHNIY, O., DI PAOLO, N. C., SILVESTRY, M., HOFHERR, S. E., BARRY, M. A., STEWART, P. L. & SHAYAKHMETOV, D. M. 2008. Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes in vivo. *Proc. Natl. Acad. Sci. U.S.A.*, 105, 5483-5488.
- KRASNYKH, V., DMITRIEV, I., MIKHEEVA, G., MILLER, C. R., BELOUSOVA, N. & CURIEL, D. T. 1998. Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. *J. Virol.*, 72, 1844-1852.
- MA, J., DUFFY, M. R., DENG, L., DAKIN, R. S., UIL, T., CUSTERS, J., KELLY, S. M., MCVEY, J. H., NICKLIN, S. A. & BAKER, A. H. 2015. Manipulating adenovirus hexon hypervariable loops dictates immune neutralisation and coagulation factor X-dependent cell interaction in vitro and in vivo. *PLoS Pathog*, 11, e1004673.

- MIZUNO, H., FUJIMOTO, Z., ATODA, H. & MORITA, T. 2001. Crystal structure of an anticoagulant protein in complex with the Gla domain of factor X. *Proc Natl Acad Sci U S A*, 98, 7230-4.
- PARKER, A. L., WADDINGTON, S. N., BUCKLEY, S. M., CUSTERS, J., HAVENGA, M. J., VAN ROOIJEN, N., GOUDSMIT, J., MCVEY, J. H., NICKLIN, S. A. & BAKER, A. H. 2009. Effect of neutralizing sera on factor x-mediated adenovirus serotype 5 gene transfer. *J Virol*, 83, 479-83.
- PARKER, A. L., WHITE, K. M., LAVERY, C. A., CUSTERS, J., WADDINGTON, S. N. & BAKER, A. H. 2013. Pseudotyping the adenovirus serotype 5 capsid with both the fibre and penton of serotype 35 enhances vascular smooth muscle cell transduction. *Gene Ther*, 20, 1158-64.
- PASQUALINI, R., KOIVUNEN, E. & RUOSLAHTI, E. 1997. Alpha v integrins as receptors for tumor targeting by circulating ligands. *Nat Biotechnol*, 15, 542-6.
- REYNOLDS, P., DMITRIEV, I. & CURIEL, D. 1999. Insertion of an RGD motif into the HI loop of adenovirus fiber protein alters the distribution of transgene expression of the systemically administered vector. *Gene Ther.*, 6, 1336-1339.
- SOUTHGATE, K. & NEWBY, A. C. 1990. Serum-induced proliferation of rabbit aortic smooth muscle cells from the contractile state is inhibited by 8-Br-cAMP but not 8-Br-cGMP. *Atherosclerosis*, 82, 113-123.
- SOYOMBO, A. A., ANGELINI, G. D., BRYAN, A. J., JASANI, B. & NEWBY, A. C. 1990. Intimal proliferation in an organ culture of human saphenous vein. *Am J Pathol*, 137, 1401-10.
- TOMKO, R. P., XU, R. & PHILIPSON, L. 1997. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc.Natl.Acad.Sci.U.S.A*, 94, 3352-3356.
- VIGNE, E., MAHFOUZ, I., DEDIEU, J. F., BRIE, A., PERRICAUDET, M. & YEH, P. 1999. RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection. *J.Virol.*, 73, 5156-5161.
- WADDINGTON, S. N., MCVEY, J. H., BHELLA, D., PARKER, A. L., BARKER, K., ATODA, H., PINK, R., BUCKLEY, S. M., GREIG, J. A., DENBY, L., CUSTERS, J., MORITA, T., FRANCISCHETTI, I. M., MONTEIRO, R. Q., BAROUCH, D. H., VAN, R. N., NAPOLI, C., HAVENGA, M. J., NICKLIN, S. A. & BAKER, A. H. 2008. Adenovirus serotype 5 hexon mediates liver gene transfer. *Cell*, 132, 397-409.
- XU, Z., QIU, Q., TIAN, J., SMITH, J. S., CONENELLO, G. M., MORITA, T. & BYRNES, A. P. 2013. Coagulation factor X shields adenovirus type 5 from attack by natural antibodies and complement. *Nat Med*, 19, 452-7.
- ZITZMANN, S., EHEMANN, V. & SCHWAB, M. 2002. Arginine-glycine-aspartic acid (RGD)-peptide binds to both tumor and tumor-endothelial cells in vivo. *Cancer Res*, 62, 5139-43.

FIGURE LEGENDS

Figure 1. RGD-4C peptide placement in HAdV-5T* and quality control analysis of purified viruses.

(a) Hexon trimer protein, arrows indicate RGD-4C insertion sites in HVR5 (orange) or HVR7R (blue). (b) HAdV-5 hexon amino acid sequence; HVR5 and HVR7 are highlighted in grey; T* point mutations are marked with asterisk (*). The amino acids removed for generation of RGD-4C replacement vectors are designated by dashed line and RGD-4C insertion point is indicated by black arrow. (c) Fiber protein, arrows indicate RGD-4C insertion sites in HI loop (purple). (d) HAdV-5 fiber amino acid sequence; arrow indicated insertion site of RGD-4C. (e) Details of vector production, indicating preparation in HEK293 cells and infectious titres measured by end-point dilution infection in HEK293 cells, physical titers quantified by microBCA and nanoparticle tracking analysis (Nanosight) and the respective VP: pfu ratios for each virus. (f) Analysis of viral proteins by SDS-PAGE and silver staining.

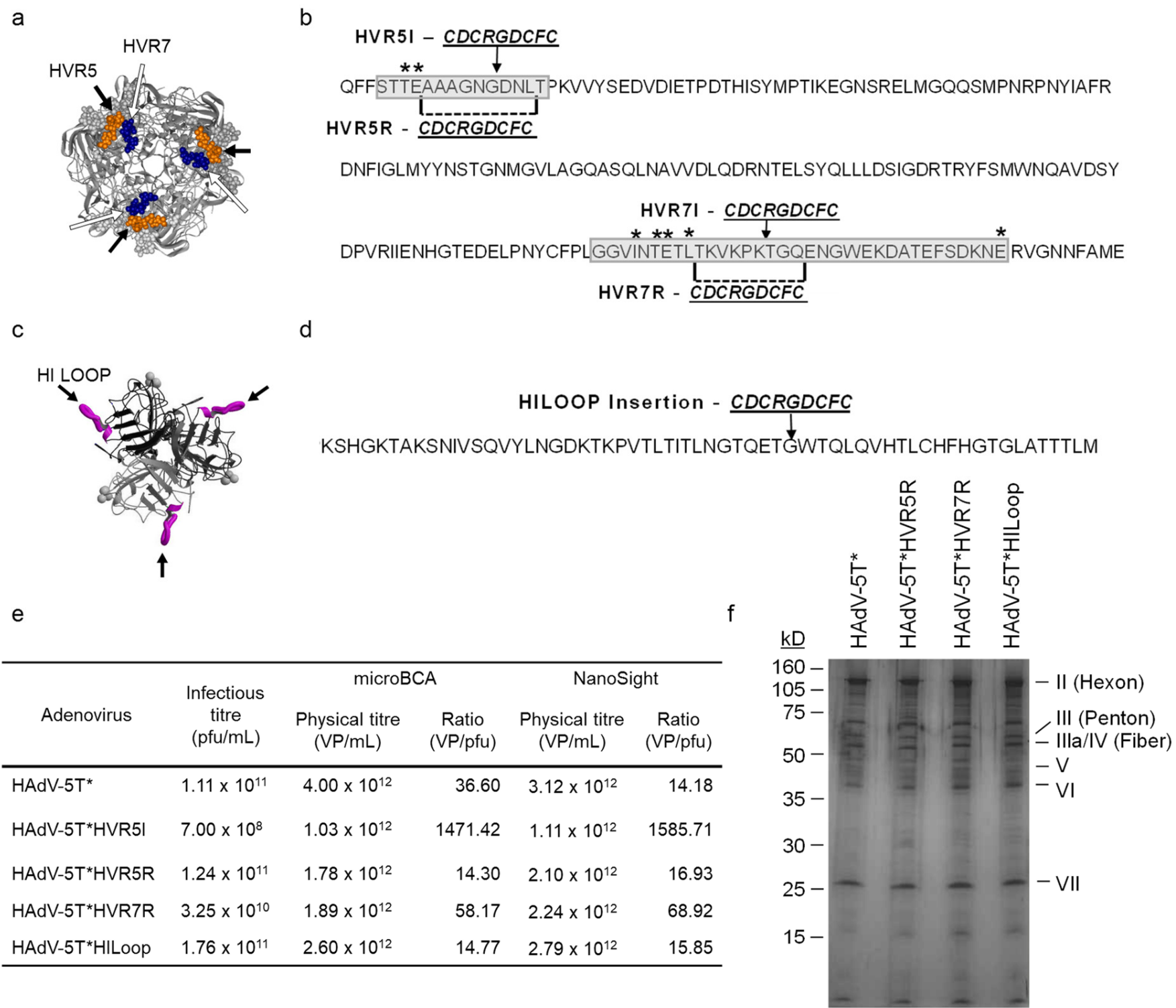
Figure 2. Assessment of HAdV-5T* retargeting by cell surface binding and viral transduction in three cell types.

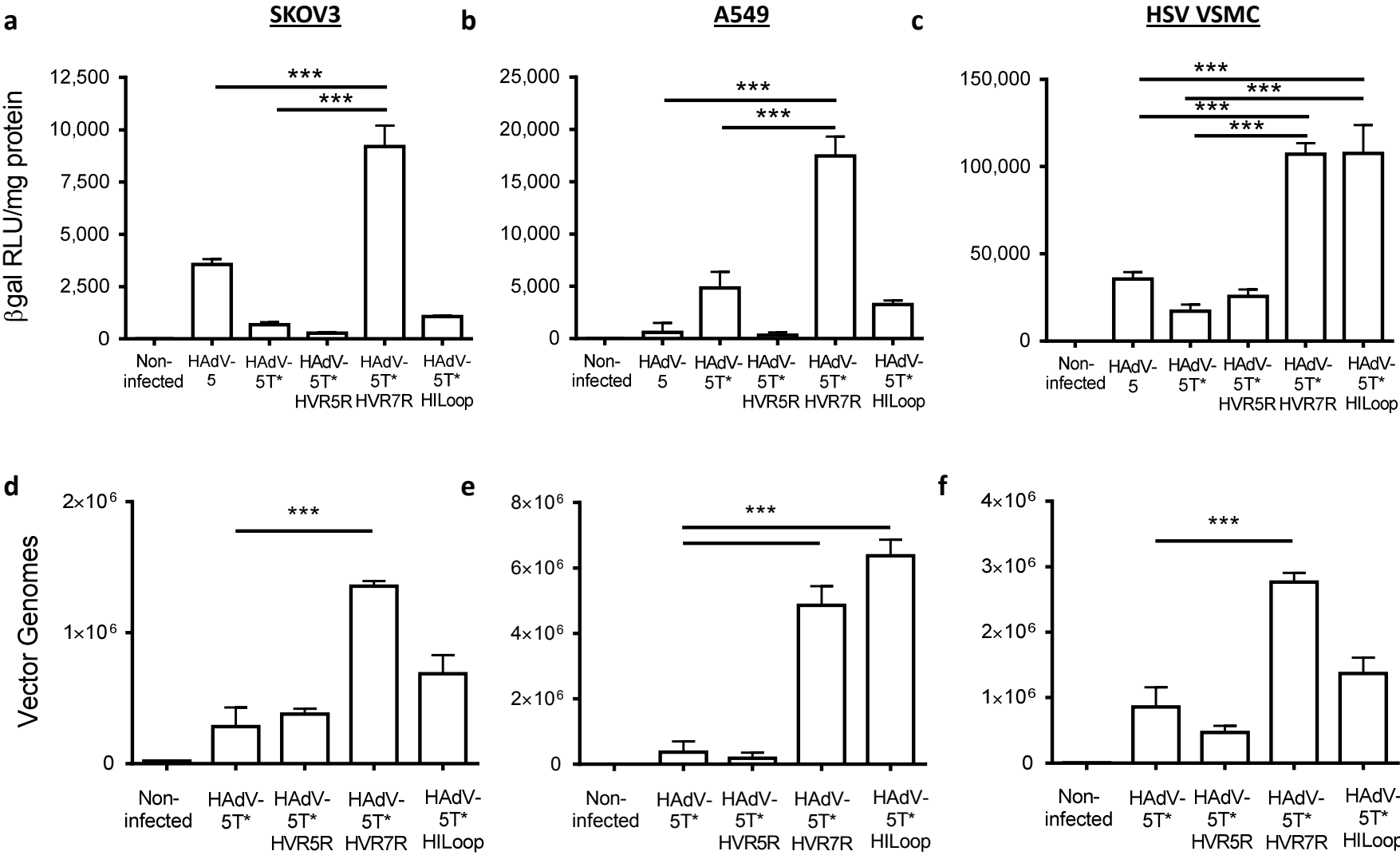
Cells were transduced with HAdV-5, HAdV-5T* and peptide-modified adenoviral vectors (HAdV-5T*HVR5R, HAdV-5T*HVR7R and HAdV-5T*HI loop) at a concentration of 50 pfu/cell for SKOV3 and A549 cells and 500 pfu/cell for human saphenous vein vascular smooth muscle cells (HSV VSMC). (a - c) Transduction was assessed 48 hours post-transduction after a 3-hour exposure to each HAdV by measuring β -galactosidase activity. (d - f) Surface binding analysis was performed by incubating the HAdV with the cells at 4°C for 1 hour and cell-bound adenoviral vectors were quantified by measuring adenoviral genomes by quantitative PCR as described in (Alba et al., 2009). Each experiment was performed in technical triplicate and repeated 3 independent times. Mean \pm Standard Deviation One-way ANOVA with Bonferroni post-hoc analysis performed using Graphpad Prism v.5; *** $p < 0.001$.

Figure 3. Evaluation of the effect of peptide insertion on evasion of neutralising anti-HAdV-5 immunity.

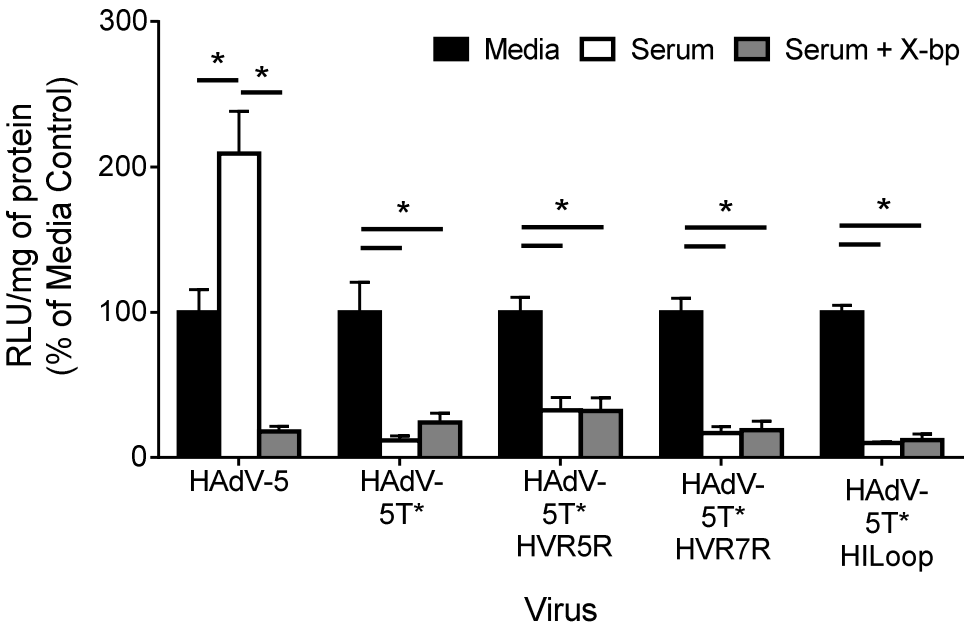
(a) HAdV-5T* and its derivatives (2×10^{10} vp/mL) were incubated with RPMI-1640 media, 90% C57BL/6 mouse serum or 90% C57BL/6 mouse serum preincubated with 40 μ g/mL X-

331 bp, for 30 min at 37°C. Virus suspensions were diluted 200-fold in serum-free media and 100
332 µL added to SKOV3 cells for 2 h at 37°C before being replaced with RPMI-1640 media with
333 2% FCS. Transgene expression was quantified 16 h post-transduction as relative light units
334 (RLU) normalized to total protein. Transduction expressed as a percentage of control
335 (HAdV-5 transduction with serum free media alone); each experiment was performed in
336 technical quadruplicate and repeated two independent times. Mean \pm SEM * $p < 0.05$. (b)
337 Effect of neutralizing sera on HepG2 cells transduction following incubation with 1,000
338 vp/cell of HAdV vectors in the presence of 1 IU/mL of FX and 2.5% sera from patients
339 previously screened for anti-HAdV-5 Nabs (Parker et al., 2009) and stained for β -Gal
340 expression 48 h post-transduction. Experiment was performed four times, data presented are
341 mean \pm SEM.





A



B

